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Differential patterns of Large Tumor Antigen specific immune responsiveness in patients with BK polyomavirus positive prostate cancer or benign prostatic hyperplasia

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Abstract: The role of the polyomavirus BK (BKV) large tumor antigen (L-Tag) as target of immune response in patients with prostate cancer (PCa) has not been investigated so far. In this study we have comparatively analyzed humoral and cellular L-Tag specific responsiveness in age matched patients bearing PCa or benign prostatic hyperplasia (BPH), expressing or not expressing BKV L-Tag specific sequences in their tissue specimens, and in non-age-matched healthy individuals. Furthermore, results from patients with PCa were correlated to 5-year follow-up clinical data focusing on evidence of biochemical recurrence (BR) following surgery (PSA 0.2ng/ml). In peripheral blood mononuclear cells (PBMC) from patients with PCa with evidence of BR and BKV L-Tag positive tumors, stimulation with peptides derived from BKV L-Tag, but not those derived from Epstein Barr virus, influenza virus or Cytomegalovirus, induced a peculiar cytokine gene expression profile, characterized by high expression of IL-10 and TGF- β 1 and a low expression of IFN- γ genes. This pattern was confirmed by protein secretion data and correlated with high levels of anti BKV L-Tag IgG. Furthermore, in PBMC from these PCa bearing patients, L-Tag derived peptides significantly expanded an IL-10-secreting CD4(+)CD25(+ (high))CD127(- (dim))FoxP3(+) T cell population with an effector memory phenotype (CD103(+)) capable of inhibiting proliferation of autologous anti-CD3/CD28 triggered CD4(+)CD25(-) T cells. Collectively, our findings indicate that potentially tolerogenic features of L-Tag specific immune response are significantly associated with tumor progression in patients with BKV+ PCa.

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Differential patterns of Large Tumor Antigen specific immune responsiveness in patients with BK polyomavirus positive prostate cancer or benign prostatic hyperplasia

Running title: BKV L-Tag specific immune response in PCa

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1 **ABSTRACT**

2 The role of the polyomavirus BK (BKV) large tumor antigen (L-Tag) as target of immune
3 response in patients with prostate cancer (PCa) has not been investigated so far. In this study we
4 have comparatively analyzed humoral and cellular L-Tag specific responsiveness in age matched
5 patients bearing PCa or benign prostatic hyperplasia (BPH), expressing or not expressing BKV
6 L-Tag specific sequences in their tissue specimens, and in non-age-matched healthy individuals.
7 Furthermore, results from patients with PCa were correlated to 5-year follow-up clinical data
8 focusing on evidence of biochemical recurrence (BR) following surgery ($\text{PSA} \geq 0.2 \text{ ng/ml}$).

9 In peripheral blood mononuclear cells (PBMC) from patients with PCa with evidence of BR
10 and BKV L-Tag positive tumors, stimulation with peptides derived from BKV L-Tag, but not
11 those derived from Epstein Barr virus, influenza virus or Cytomegalovirus, induced a peculiar
12 cytokine gene expression profile, characterized by high expression of IL-10 and $\text{TGF}\beta$ -1 and a
13 low expression of IFN- γ genes. This pattern was confirmed by protein secretion data and
14 correlated with high levels of anti BKV L-Tag IgG. Furthermore, in PBMC from these PCa
15 bearing patients, L-Tag derived peptides significantly expanded an IL-10-secreting
16 $\text{CD4}^+ \text{CD25}^{+(\text{high})} \text{CD127}^{-(\text{dim})} \text{FoxP3}^+$ T cell population with an effector memory phenotype
17 (CD103^+) capable of inhibiting proliferation of autologous anti-CD3/CD28 triggered $\text{CD4}^+ \text{CD25}^-$
18 T cells. Collectively, our findings indicate that potentially tolerogenic features of L-Tag specific
19 immune response are significantly associated with tumor progression in patients with BKV+
20 PCa.

21 **KEY WORDS**

22 Human Polyomavirus BK Large Tumor Antigen, Prostate Cancer, Regulatory T cells, Viral
23 Immunology.

1 INTRODUCTION

2 Prostate cancer (PCa) represents the first leading cause of cancer morbidity and the third
3 of cancer death in men in developed countries with a worldwide incidence rate of 14% of total
4 newly diagnosed malignancies and a worldwide total cancer mortality rate of 6% (29). A
5 contemporary model of PCa induction and progression should include the analysis of the
6 contribution of inflammation to the development of preneoplastic or neoplastic lesions (24).
7 Indeed, proliferative inflammatory atrophy (PIA) of the prostate has recently gained importance
8 as potential precursor of prostatic intraepithelial neoplasia (PIN) and overt PCa (15), (14). This
9 owes particularly to the prevalence of PIA in the peripheral zone of the organ, where histological
10 transition between PIA and PIN usually occurs (42).

11 The low rate of mutations detected in tumor suppressor genes pRB1 and p53 in primary PCa
12 cells (16) has suggested a possible role of inflammatory agents ubiquitous in the urinary tract
13 with unique oncogenic functions, that is of sequestering wild-type products of tumor suppressor
14 genes. Polyomaviruses' main regulatory proteins large tumor antigens (L-Tag) interfere with wt-
15 p53 binding to cellular DNA during virus infection (4), thereby impairing p53 control on cell
16 growth activity and possibly leading to oncogenic transformation in non-permissive cells (6). As
17 such, the human urotheliotrophic polyomavirus BK (BKV) has been suggested to prominently
18 associate with the development of cancer (26, 27), and, in particular, of urinary tract
19 malignancies (3, 12, 54).

20 There is a continuing debate on BKV expression in overt cancers (1, 5). However, the
21 possibility of "hit and run" carcinogenic mechanisms induced by BKV cannot be excluded (13).
22 Genetically rearranged BKV variants (45), (22), presumably difficult to detect by commonly used
23 assays, might exist in the urinary tract and be responsible for neoplastic transformation in prostate

cells (i.e. URO-1) (37). Therefore, BKV has been indicated as potential co-factor in the earliest stages of PCa (13).

Detection and expression of BKV L-Tag sequences in preneoplastic prostate tissues (12) have prompted us to investigate the role of this viral antigen as target of immune response. Thus, in this study we have addressed humoral and cellular responsiveness to BKV L-Tag in patients with benign prostatic hyperplasia (BPH) or newly diagnosed PCa and we have correlated viral and immunological features with PCa status.

PATIENTS & METHODS

Patients and clinical follow-up

One hundred ten consecutive patients diagnosed for either prostate cancer (PCa, n=60; PSA>4ng/ml and suspicious digital rectal examination (DRE) and/or positive for early diagnosed high grade prostate intraepithelial neoplasm (HGPIN) at biopsy) or benign prostate hyperplasia (BPH n=50; urinary obstructive symptoms and acute urinary retention according to the International Prostate Symptom Score, IPSS) were enrolled in the study at the Department of Urology of the University Hospital of Basel, Switzerland, and at the Division of Urology of the University Hospital of Zurich, Switzerland, upon informed consent, following approval by Cantonal Ethical Committees of Basel and Zurich. Five-year follow-up after surgery was completed for 48/60 PCa patients (80%). Timing of biochemical recurrence (BR+), with early censoring if only 1 or 2 values were available, was established at the first ≥ 0.2 ng/ml PSA detection. BR- patients were those showing clearly negative PSA values (< 0.04 ng/ml) during complete follow-up. Data were collected according to the American Society for Therapeutic Radiation and Oncology (ASTRO) criteria (23), considering both ACTUARIAL and ASTRO-time analytical methods, since ASTRO-censoring allowed to define biochemical failure by 3 consecutive increases in PSA values in 3/48 PCa only.

Virus detection in tissue specimens

Specimens from tissues excised during surgical procedures were formalin-fixed and paraffin-embedded. DNA was extracted from 3 sections (thickness, 5 μ m) randomly picked within the tumor area (PCa) or within the atrophic-hyperplastic gland (BPH) using the QIAamp DNA Mini Kit (Qiagen, Basel, Switzerland), according to the manufacturer's instructions.

Molecular detection of polyomavirus BK was performed by qRT-PCR using a TaqMan assay targeting L-Tag. The assay, named T3a, consists of a primer/probe set which has been designed to reliably measure BKV L-Tag subtypes Ia, Ic, III, IV and VI (25). PCR amplification was set up according to standard real-time PCR protocols, using a Corbett life science Rotor-gene 3000 instrument (Corbett Life science, Sydney, Australia). Standard curves for the quantification of BKV L-Tag were generated using serial 10-fold dilutions of plasmid pBKV35-1 DNA (LGC Standards Sarl, Molsheim, France). To correct for the variable amounts of DNA in individual tissue specimens, amplification of control gene aspartoacylase (ACY) was performed in each sample, as previously described (43). Patients were considered BKV L-Tag negative upon consecutive negative testing on all three randomly picked punches.

Enzyme Immunoassay (EIA) for patient and donor serology

BKV specific antibody detection was performed by enzyme immunoassay (EIA) with GST-BKV fusion protein. Optical densities (OD₄₉₂) were measured using an automated plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 492 nm. Affinity-purified GST was run as a negative control and corresponding OD values were subtracted from GST-BKV L-Tag domain 1 (LTD1) and GST-BKV VP1 specific signals. The cut-off was defined as two standard deviations above mean values of negative controls. Therefore, all OD values <0.04 were considered negative (7, 33).

Peptides and peptide pools

BKV Large T antigen (L-Tag) peptide-pool was provided by JPT Peptide Technology (Berlin, Germany). This PepMix contains a total of 170 15mer peptides spanning the entire

antigen (691 amino acids, Swiss-Prot P14999) and tiled at 11 amino acid pace. Similarly, the negative control PepMix HIVgag peptide-pool (123 peptides including B gag motif) was also provided by JPT. As positive control, the PepMix CEF (Cytomegalovirus, Epstein Barr virus and Influenza virus) pool of 23 8-11mer peptides recognized by CD8 positive T cells and presented by 11 class I HLA-A and HLA-B alleles (11) was used (JPT, Berlin Germany). For *in vitro* expansion and proliferation assays, both HIV peptide-pool (see above) and the Human Cytomegalovirus promiscuous epitope pp65₃₄₀₋₃₅₅ (41) (Princeton Biomolecules, Langhorne, PA, USA) were used as controls.

“*Ex vivo*” induction and detection of cytokine gene expression by qRT-PCR

Peripheral blood mononuclear cells (PBMC) isolated from venous blood by Ficoll-Hypaque density gradient centrifugation were resuspended in RPMI medium supplemented with 100 µg/ml Kanamycin, 10 mM Hepes, 1mM sodium pyruvate, 1 mM Glutamax and nonessential amino acids (all from GIBCO Paisley, Scotland) (complete medium) and 5% human serum (Blutspendezentrum UniversitätsSpital Basel, Switzerland), at a final concentration of 1×10^6 cells/ml, plated in 96 U-bottom well plates (200 µl/well) and incubated for overnight resting. Cells were then stimulated with either test (L-Tag) or control (CEF, HIV) peptide-pool (1 µg/ml) or PHA (1 µg/ml) and harvested after 3 hours for RNA extraction (RNeasy[®] Mini Kit Protocol, Qiagen, Basel, Switzerland) and cDNA synthesis (Invitrogen, Carlsbad, CA, USA). Quantitative gene amplification (qRT-PCR) was performed, as previously described (40), by an ABI prism[™] 7500 FAST sequence detection system using TaqMan[®] Universal PCR Master Mix Reagents Kit and “on demand” sets of primers and probes for cytokine gene expression (IFN-γ, IL-10, TGF-

1 β 1) (Applied Biosystems, Rotkreuz, Switzerland). β -actin was used as endogenous reference
2 gene and normalized data were analyzed by the $2^{-\Delta\Delta C_t}$ method (34).

4 **Cell cultures**

5 Monocytes (CD14⁺) sorted from PBMC by magnetic beads (Miltenyi Biotech) were
6 cultured from 5 to 7 days in RPMI complete medium supplemented with 10% FCS (GIBCO
7 Paisley, Scotland), 0.004% β -mercaptoethanol, rhIL-4 (1000 U/ml) and rhGM-CSF (50 ng/ml) to
8 generate immature DCs (iDCs). To induce maturation, iDCs were overnight exposed to 1 μ g/ml
9 LPS (Abortus Aequi, Sigma-Aldrich, St. Louis, MO, USA). CD4⁺ T cells positively sorted from
10 PBMC by magnetic beads were cultured in complete medium with 5% human serum in 24 flat-
11 bottom well plates at a 1×10^6 cells/ml concentration in the presence of autologous irradiated
12 mature DC (mDCs), previously pulsed for 2 hours with peptide pools (10 μ g/ml), either for
13 priming (day 0) or re-stimulation (day 7). Recombinant human IL-2 (rhIL-2; Hoffmann-LaRoche,
14 Basel, Switzerland) was added to cultures at 1ng/ml, 1ng/ml and 5ng/ml, on days 3, 7 and 10,
15 respectively.

16 Regulatory T cell cultures were established by *ex vivo* magnetic sorting of
17 CD4⁺CD25⁺CD127^{-dim} subset, as previously described (48). Cells were subsequently stimulated
18 with L-Tag peptide-pool (5 μ g/ml) or control peptides, including HCMVpp65₃₄₀₋₃₅₅ and HIV
19 peptide-pool, in the presence of 10 μ g/ml anti-CD28 (BD Bioscience, Allschwil, Switzerland)
20 and 5 ng/ml of rIL-2. Cultures were similarly restimulated on day 7. IL-10-secreting Treg were
21 sorted on day 14 by cytokine secretion assay (Miltenyi Biotec, Germany). For suppression
22 assays, CD4⁺CD25^{+(high)} CD127^{-(dim)}, IL-10-secreting T cells were cultured in the upper chamber
23 of 24-transwell plates (BD Bioscience, Allschwil, Switzerland) with a double amount

(ratio=0.5:1) of autologous CD4⁺CD25⁻ T cells, stimulated with 1mg/ml of anti CD3/CD28 (BD Bioscience, Allschwil, Switzerland) seeded in the lower chamber, and cultured for 8 days. CD4⁺CD25⁻ T cells proliferation was measured using Cell Proliferation Reagent WST-1 (Roche Applied Science, Rotkreutz, Switzerland) according to manufacturer's instructions and absorbance was read at 450nm with an ELISA plate reader.

Regulatory T cell quantification by flow cytometry

CD4⁺ T cells stimulated by peptide-pools, as described above, were stained with anti-CD4 FITC and anti-CD25 APC antibodies (BD Biosciences, Allschwil, Switzerland). Anti FoxP3 PE antibodies and FoxP3 Fix/Perm buffer were used for intracellular staining according to manufacturer's protocol (eBioscience, Vienna, Austria). Separation into CD25 bright (CD25^{high}) and CD25 dim (CD25^{low}) cells was carried out using a fluorescence cut-off defined in healthy donors (HD). Anti-CD103 PerCP (eBioscience, Vienna, Austria) and anti-IL-10 APC intracellular staining (BD Bioscience, Allschwil, Switzerland) were additionally carried out for the identification of IL-10-secreting activated Treg. Data were acquired on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose`, CA, USA).

Cytokine measurement

The FlowCytomix Simple kit (eBioscience, Vienna, Austria) was used to measure IFN- γ , IL-10 and TGF- β 1 protein release in culture supernatants of peptide-stimulated T cells. Samples were run on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose`, CA, USA), analyzed with BMS FlowCytomix software (eBioscience, Vienna, Austria), and referred to a standard curve for quantification.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (version 5.1) and SAS/STAT (version 9.1). Data were reported as mean \pm standard deviations (SD) or mean \pm standard errors (SE), median and ranges where appropriate. Distributions of categorical markers were analyzed by both χ^2 and Fisher's exact tests. Groups following normal distribution (Shapiro-Wilk test) were compared with *t*-tests (either Pooled or Cochran method) and *F*-test was used to calculate variance among groups of samples. If non-normal distribution was indicated, nonparametric tests such as Mann-Whitney U tests and Spearman's ρ correlation analyses were used. *P* values <0.05 (CI 95%) were considered statistically significant.

RESULTS

Clinical profiles of patients

The average age of patients enrolled in the study (n=110) was 64 ± 7.7 years (range 49-90) for PCa (n=60) and 67 ± 7.4 years (range 48-81) for age-matched BPH (n=50), respectively.

Out of 60 PCa patients, 1 was diagnosed with clinical stage pT1b, 10 with pT2a, 2 with pT2b, the majority (n=30; 51%) with clinical stage pT2c, 4 with pT3a and 3 with pT3b. Ten (17%) patients had tumors whose stage at diagnosis could not be assessed. Average serum prostate specific antigen (PSA) value was 17.4 ± 36.2 ng/ml (median: 6.7 ng/ml, range: 1.6-206 ng/ml) in PCa patients before surgery (n=50, 83%) and 5.6 ± 5.7 ng/ml (median: 3.9 ng/ml, range: 0.4-26 ng/ml) in BPH patients (n=36, 72%). Gleason Score (GS) of tumor specimens (n=55, 92%) ranged from 5 to 9 according to immunohistochemical analysis (Table 1).

L-Tag DNA detection in tissue specimens and BKV specific humoral response

Presence of polyomavirus BK was analyzed by BKV L-Tag DNA detection in surgically excised PCa (n=43/60, 72%) and BPH (n=38/50, 76%) specimens. Positive samples (hereafter referred to as BKV+) were identified among both PCa (mean=451.4 copies/ 10^5 cells; n=18/43, 42%) and BPH (mean=391.5 copies/ 10^5 cells; n=12/38, 32%) with no significant quantitative differences among groups of patients (p=0.72) (Figure 1a). No significant association (χ^2) with cancer (p=0.18) or Gleason Score (GS+ ≥ 7 ; p=0.82) was observed. However, high PSA levels (PSA ≥ 4 ng/ml) were more frequently detectable in sera from patients with BKV+ PCa (p<0.05).

Humoral response against BKV VP1- and L-Tag was analyzed in sera from patients with PCa and BPH. Upon sample stratification based on BKV L-Tag DNA tissue testing, we noted that levels of L-Tag specific IgG (OD) were significantly higher in patients bearing BKV+ than BKV-

PCa ($p=0.01$), while they were similarly low in patients with BKV+ or BKV- BPH. In contrast, anti VP1 IgG levels were similar in patients with PCa or BPH, irrespective of their BKV molecular status (Figure 1b). Overall, detectable evidence of humoral responsiveness to L-Tag ($OD \geq 0.04$) was observed in 17/18 (94%) patients with BKV+ PCa and in 7/12 (58%) patients with BKV+ BPH with a significant association (χ^2) between BKV+ lesions and L-Tag IgG only in patients with PCa ($p=0.005$) (Table 1).

Cytokine gene expression pattern in PBMC from patients with PCa or BPH upon *ex vivo* BKV L-Tag peptide-pool stimulation

Humoral response data, showing a high level of L-Tag IgG response in patients bearing BKV+ PCa, prompted us to explore cellular immune responsiveness to this antigen. Freshly isolated PBMC from 60 patients with PCa and 50 age-matched patients with BPH were 3-hour *ex vivo* stimulated with an L-Tag peptide-pool and IFN- γ and IL-10 cytokine gene expression were used as readout. A HIV peptide-pool with B gag motif was used as negative control to provide a baseline allowing the calculation of cytokine gene expression fold changes.

In L-Tag IgG seronegative patients (PCa $n=14$, BPH $n=12$, Table 1), regardless of underlying disease, L-Tag peptide-pool stimulated cytokine gene expression was usually low (Figure 2a). However, in L-Tag IgG+ patients with PCa ($n=46$), L-Tag peptide-pool induced a significantly higher IL-10 gene expression, ($p=0.03$), but rather a decreased IFN- γ gene expression ($p=0.04$) as compared to seronegative patients. In contrast, in L-Tag IgG+ patients with BPH ($n=38$) L-Tag peptide-pool did not induce significantly higher IFN- γ ($p=0.8$) and IL-10 ($p=0.9$) gene expression, as compared to seronegative patients. Accordingly, these tests also showed that L-Tag peptide-pool induced a higher IL-10 than IFN- γ gene expression in L-Tag IgG+ patients with

1 PCa (n=46; p=0.0001) and, to a lesser, non-significant, extent, in L-Tag IgG+ patients with BPH
2 (n=38; p=0.06) (Figure 2a).

3 To investigate the relationship eventually occurring between BKV L-Tag specific humoral
4 response and L-Tag-specific gene expression pattern, L-Tag specific IgG activity was plotted
5 against IL-10/IFN- γ gene expression ratio. A significant direct correlation was indeed observed in
6 patients with PCa (Spearman: $\rho=0.4$, p=0.009), but not in patients with BPH ($\rho=-0.2$, p=0.3)
7 (Figure 2b).

8 These results urged us to comparatively investigate cytokine gene expression induced by L-
9 Tag peptide-pool, CEF peptide-pool or PHA in L-Tag seropositive patients with PCa or BPH and
10 in seropositive gender but not age-matched healthy donors (HD). Contrary to PBMC from IgG+
11 patients with PCa and BPH (see above), L-Tag peptide-pool stimulation of PBMC from L-Tag
12 IgG+ HD induced a IFN- γ gene expression significantly higher than IL-10 gene expression (n=8;
13 p=0.003). Most importantly, CEF peptide-pool and PHA induced a similar, significantly higher
14 IFN- γ than IL-10 gene expression in PBMC from patients with PCa or BPH (p<0.0001) or from
15 HD (p<0.001) (Figure 2c).

16 Notably, IL-10 gene expression induced by L-Tag peptide-pool was significantly higher
17 than IL-10 gene expression induced by CEF or PHA in PBMC from L-Tag IgG+ patients with
18 PCa (p<0.0001 and p=0.0002, respectively) or BPH (p<0.0001 and p=0.001, respectively)
19 (Figure 2c). In PBMC from HD, IL-10 gene expression was similarly negligible irrespective of
20 the stimuli used.

21 Remarkably, IFN- γ gene expression could be induced by CEF or PHA to comparably high
22 extents in PBMC from L-Tag IgG+ patients with PCa or BPH or from HD (Figure 2c), thereby

1 suggesting a specificity of the cytokine gene expression pattern induced by L-Tag stimulation in
2 PBMC from L-Tag IgG+ patients.

3 4 **BKV L-Tag peptide-pool stimulated cytokine gene expression pattern in PBMC from** 5 **patients bearing BKV+ PCa or BPH**

6 We then evaluated in detail L-Tag specific cellular immune response in patients bearing
7 BKV+ or BKV- PCa or BPH. The analysis of these data confirmed that a highly significant
8 increase in IL-10 gene expression upon L-Tag peptide-pool stimulation was detectable in PBMC
9 from patients bearing a BKV+ PCa as compared to those from patients bearing BKV- PCa
10 ($p < 0.0001$). In contrast, IFN- γ gene expression induced by L-Tag peptide-pool was modest, but
11 detectable in PBMC from patients with BKV- PCa, and significantly higher than that detectable
12 in similarly stimulated PBMC from patients bearing BKV+ PCa ($p = 0.02$) (Figure 3a). On the
13 other hand, expression of IFN- γ and IL-10 genes upon L-Tag peptide-pool stimulation was
14 similar in PBMC from patients bearing BKV+ or BKV- BPH.

15 An analysis limited to patients with evidence of BKV L-Tag specific IgG also showed a
16 highly significant ($p < 0.0001$) predominance of IL-10 vs. IFN- γ gene expression in patients
17 bearing BKV+ prostate lesions. In contrast, IFN- γ and IL-10 gene expression detectable upon L-
18 Tag triggered PBMC stimulation were similar in L-Tag IgG+ patients with BKV+ BPH (Figure
19 3b).

20 Indeed, although IL-10 gene expression did not significantly differ between patients with
21 BKV+ PCa or BKV+ BPH ($p > 0.05$) (Figure 3a and 3b), most interestingly, IL-10/IFN- γ gene
22 expression ratio was significantly ($p = 0.04$) higher in BKV specific IgG+ patients bearing BKV+
23 PCa ($n = 17$) than in BKV specific IgG+ patients with BKV+ BPH ($n = 6$) (Figure 3c).

Comparative analysis of L-Tag induced cytokine gene expression in patients bearing BKV+ PCa with or without evidence of biochemical recurrence (BR).

The results obtained in the first part of our study suggested that BKV specific immune response is characterized by specific features, particularly in patients bearing BKV+ PCa, as compared to patients with BPH, let alone healthy donors. These data raised the obvious issue of the association of this response pattern with PCa clinical course.

Therefore, considering their peculiar clinical condition, we analyzed in detail BKV L-Tag peptide-induced cytokine gene expression in PBMC from patients bearing BKV+ cancers with biochemical evidence of recurrence (BR+). BR was observed in 9/48 (19%) patients with PCa with complete follow-up, after 6 weeks (n=6), 24 weeks (n=1), 48 weeks (n=1) and 96 weeks (n=1). Two patients were excluded from the study because BKV molecular testing on tissue lesions was not available (*nt*) (Table 2). Despite the limited number of BR+ PCa patients, a significant association between BR status and BKV+ lesions, irrespective of serological status, was observed. Indeed, in 7/16 patients bearing BKV+ PCa, evidence of BR was detectable within a 96 weeks follow up, as compared to none of 17 patients with BKV- PCa (Fisher exact test, $p=0.004$).

Then, we comparatively analyzed cytokine gene expression upon L-Tag stimulation in patients with BKV+ PCa stratified based on evidence of BR. In order to evaluate a more homogeneous test group, we excluded the only BR- patient with BKV+ PCa, seronegative for L-Tag specific IgG (Table 2).

In BKV L-Tag specific IgG+ patients with BKV+ PCa (n=15), a distinctive L-Tag induced gene signature prominently emerged. Indeed, significantly higher IL-10/IFN- γ ($p<0.01$) and TGF- β 1/IFN- γ ($p=0.02$) gene expression ratios were observed in PBMC from patients bearing a

BKV+ PCa with BR evidence (n=7), as compared with those detectable in PBMC from patients bearing a BKV+ PCa with no BR evidence (n=8) (Figure 4a).

Importantly, in the former group, the extent of L-Tag specific IgG response was significantly correlated with both the IL-10/IFN- γ ($p=0.8$, $p=0.02$) and TGF- β 1/IFN- γ ($p=0.7$, $p=0.05$) gene expression ratio induced in PBMC by L-Tag peptide stimulation (data not shown).

To verify these findings at the protein level, we cultured CD4⁺ T cells from patients bearing BKV+ PCa with (n=7) or without (n=8) evidence of BR in the presence of autologous DC pulsed with either HIV derived or L-Tag peptide pools and we analyzed IFN- γ , IL-10 and TGF- β 1 release upon specific stimulation. In keeping with gene expression data (see above), we found that L-Tag specific stimulation in BR+ patients with BKV+ cancers induced a significantly ($p<0.01$) lower IFN- γ production and a significantly ($p=0.03$) higher TGF- β 1 production, as compared to cells from BR- patients bearing BKV+ PCa (figure 4b). Although IL-10 release did not significantly differ in the two groups, IL-10/IFN- γ protein ratios were significantly ($p<0.01$) higher in BR+ patients bearing BKV+ PCa.

L-Tag peptide-pool stimulation expands CD4⁺CD25^{+(high)}FoxP3⁺ T cells in patients bearing BKV+ PCa with biochemical recurrence

The peculiar gene expression pattern induced by L-Tag in patients bearing BKV+ PCa, and, particularly, in BR+ patients, prompted us to investigate CD4⁺ regulatory (CD25⁺FoxP3⁺) T cells in PBMC from these patients, both *ex vivo* and following L-Tag peptide-pool-specific stimulation. In particular, to adequately control our study, we focused on cells from L-Tag specific IgG+ patients bearing BKV+ PCa with or without BR (n=7 and n= 8, respectively) and from L-Tag specific IgG+ patients bearing BKV+ BPH (n=7) (Table 2).

Peripheral blood CD4⁺ T cells from these patients were stained on their surface with anti CD25 mAb and intracellularly with anti Foxp3 mAb. No differences were detectable between patients with PCa, irrespective of BR, and patients with BPH regarding CD4⁺CD25⁺FoxP3⁺ T cells *ex vivo* frequency (Figure 5a).

CD4⁺ T cells from the different groups of patients under investigation were then cultured for 2-weeks in the presence of L-Tag pool, HIV pool or HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide-pulsed autologous mDCs. CD4⁺CD25^{+(high)} populations in cultured cells were identified as shown in figure 5b. Following L-Tag peptide-pool stimulation, cultures from BR+ patients bearing BKV+ PCa contained significantly higher percentages of CD4⁺CD25^{+(high)}FoxP3⁺ cells, as compared to similarly stimulated cultures from BR- patients bearing BKV+ PCa (p<0.001) or to cultures from patients bearing BKV+ BPH (p<0.001) (Figure 5c). In contrast, HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide failed to expand CD4⁺CD25^{+(high)}FoxP3⁺ cells to extents significantly higher than HIV pool peptides in cells from any of the patient population under investigation. Furthermore, FoxP3⁺ protein expression upon L-Tag, but not HCMVpp65₃₄₀₋₃₅₅ or HIV pool peptide stimulation, was significantly enhanced, as evaluated by mean fluorescence intensity (MFI) measurement, in cells from BR+ patients bearing BKV+ PCa in comparison to cells from BR- patients bearing BKV+ PCa (p<0.001) or from patients bearing BKV+ BPH (p=0.001) (Figure 5d).

Functional effector memory phenotype (CD103) and regulatory activity of BKV L-Tag *in vitro* generated CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells

To investigate the potential functional relevance of the phenotypic modifications observed upon L-Tag stimulation, PBMC from BR+ patients bearing BKV+ PCa (n=3) were cultured in

1 the presence of L-Tag peptide-pool, HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide and HIV peptide-
2 pool, as detailed in “materials and methods”. CD4⁺CD25^{+(high)} T cells were then sorted as CD127⁻
3 ^(dim) and incubated in the presence of the antigens used for initial stimulation. High percentages of
4 L-Tag and HCMVpp65₃₄₀₋₃₅₅ stimulated CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells were able to produce
5 IL-10, as detectable by intracellular staining (99.1±0.2% and 78.9±3.2%, respectively) (figure
6 6a). However, L-Tag stimulated CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells showed a higher expression
7 of CD103, a Treg effector memory marker (35), as compared to HCMVpp65₃₄₀₋₃₅₅ or HIV
8 peptide-pool stimulated cells (Figure 6b). Most importantly, L-Tag expanded
9 CD4⁺CD25^{+(high)}CD127^{-(dim)}, IL-10-secreting T cells were able to inhibit the proliferation of
10 autologous anti-CD3/CD28 stimulated CD4⁺CD25⁻ T cells, in co-culture assays at a 0.5:1 ratio,
11 to a significantly higher extent than HCMVpp65₃₄₀₋₃₅₅ expanded CD4⁺CD25^{+(high)}CD127^{-(dim)} T
12 cells (p<0.0001) (Figure 6c and 6d).

Discussion

BKV infection has repeatedly been suggested to be associated with cancers of the genitourinary tract. However, possibly due to conflicting results regarding detection of specific sequences and proteins in human cancers, its oncogenic role is controversial (1).

In a recent past, we have analyzed in detail cellular immune responses to BKV L-Tag derived antigenic epitopes (39). Prompted by these studies we have now addressed key features of BKV L-Tag specific immune responsiveness in patients with PCa, and their association with clinical course.

Our data unravel that in patients with PCa, and, in particular, in those showing biochemical evidence of tumor recurrence (BR+), immune responsiveness to BKV L-Tag is differentially characterized by a number of conspicuous features, as compared with patients with no evidence of BR, or patients with BPH.

Indeed, in patients with BR+ PCa, a high titer of L-Tag specific IgG is significantly associated with a high IL-10/IFN- γ gene expression ratio as observed following *ex vivo* PBMC stimulation with L-Tag peptides. Furthermore, in these patients, L-Tag peptide stimulation of PBMC *in vitro* results in a significantly higher expansion of a CD4⁺CD25^{+(high)}FoxP3⁺ population as compared with patients bearing BKV+ PCa without BR evidence or with patients with BKV+ BPH. Notably, our data indicate that BKV L-Tag gene expression is detectable with similar frequency and at similar copy numbers in PCa and BPH tissues. Thus, we did not detect a preferential BKV gene expression in PCa (5), but rather a different pattern of L-Tag specific immune responses.

At difference with capsid proteins, polyomavirus L-Tag is not present in viral particles, but is only produced in infected cells and localizes in their nuclei. Therefore, while humoral response to

1 capsid proteins is widely detectable following infection, induction of L-Tag specific humoral
2 responses is less likely to take place, unless cell death repeatedly occurs, which has been suggested
3 to be the case in cancer tissues (38). Alternatively, we might hypothesize that only BKV abortive
4 infections, e.g. those possibly leading to cancer transformation of infected cells, would permit L-
5 Tag exposure to the immune system, thus favoring the induction of an antibody response (38).
6 Indeed, our data show a significant association between L-Tag specific IgG levels and L-Tag
7 molecular detection in patients with PCa, but not in those with BPH. In contrast, VP1 IgG levels
8 were similar in patients with PCa and BPH, irrespective of BKV detection in prostate lesions,
9 partially reflecting humoral responses observed against Merkel cell polyomavirus (MCV) VP1 in
10 Merkel Cell Carcinoma (MCC), a cancer associated to MCV infection (50).

11 Cellular immune responsiveness to BKV L-Tag is also characterized by a number of peculiar
12 features. In particular, in PBMC from patients with PCa, L-Tag peptide specific response is
13 characterized by an IL-10 gene expression significantly higher than that of IFN- γ gene. In PBMC
14 from patients with BPH a similar trend is detectable, whereas in cells from HD, IFN- γ gene
15 expression is significantly higher than that of IL-10 gene. This gene expression pattern is unique
16 to BKV L-Tag stimulated cells, because in PBMC from patients with PCa, BPH or from HD,
17 other viral peptides (CEF pool) or PHA similarly induce an IFN- γ gene expression significantly
18 higher than that of IL-10 gene. Therefore, the skewed responsiveness to BKV L-Tag cannot be
19 merely attributed to a physiological decline of immune fitness, as frequently occurring in elderly
20 individuals.

21 Taken together, these data suggest that the analysis of systemic BKV seroprevalence, as
22 determined by IgG activity against viral capsid proteins, fails to support an association between
23 BKV infection and PCa (38). Instead, humoral, and, most of all, cellular responsiveness to BKV

1 L-Tag skewed towards an immune regulatory gene expression profile, and characterized by a
2 high IL-10/IFN- γ gene expression ratio, appear to be associated with PCa. Most conspicuously,
3 this pattern of responsiveness is typically detectable in cells from patients with recurrent PCa.

4 Interestingly, recent data indicate that PBMC from patients with MCC might fail to produce
5 IFN- γ in response to specific L-Tag peptides (28). In this case as well, infection by MCV was
6 found to induce L-Tag specific humoral responses predominantly in cancer bearing patients.
7 However, L-Tag stimulated IL-10 production was not explored.

8 It is tempting to speculate that the skewed cytokine gene expression signature typically
9 detectable upon L-Tag peptide stimulation could be due to immune regulatory activities of cells
10 preferentially secreting IL-10. However, while IFN- γ is only produced by T and NK cells, IL-10
11 can be produced by several cell types, including tumor cells (10, 21), virally infected cells (9),
12 different types of activated antigen presenting cells and T cells.

13 We found similar numbers of circulating cells expressing classical CD4⁺CD25^{+(high)}FoxP3⁺
14 Treg phenotype in patients with PCa or BPH, irrespective of their BR and L-Tag molecular
15 status. However, our data indicate that *in vitro* stimulation of PBMC from patients with recurrent
16 BKV+ PCa, but not from patients with BKV+ PCa and no BR evidence or from patients with
17 BKV+ BPH, results in the significant expansion of cells characterized by a typical Treg
18 phenotype. These lymphocytes uniformly express IL-10, as detectable by intracellular staining,
19 consistent with a predominant role in the elicitation of the skewed cytokine gene expression
20 profile induced by BKV L-Tag in PBMC from patients bearing BKV+ PCa. Admittedly, the sole
21 expression and/or release of IL-10 does not represent a reliable signature for regulatory T cell
22 involvement (44) since it can be also induced in effector cells to reduce inflammation at the site
23 of acute viral infections (49). However, the detection of this cytokine in combination with both

1 TGF- β 1 and permanent FoxP3 expression is usually associated to regulatory functions (2, 52).
2 Most importantly, L-Tag induced CD4⁺CD25^{+(high)}FoxP3⁺CD103⁺ T cells also display a
3 remarkable capacity to inhibit proliferation of autologous anti-CD3/CD28 stimulated CD4⁺CD25⁻
4 lymphocytes as compared to HCMVpp65 stimulated CD4⁺CD25^{+(high)}FoxP3⁺CD103^{+/-} T cells, in
5 accordance to recently published reports (46, 56). Collectively, these data hint to mechanisms
6 potentially underlying our findings in *ex vivo* activated PBMC.

7 Immune regulatory activities driven by viral antigens and promoting the establishment of
8 chronic infections have already been described (18, 20). These functions can be elicited by
9 recruiting either CD4⁺ or CD8⁺ Treg, depending on type of infections (30). The involvement of
10 both Treg subsets is dictated by HLA-restricted virus-specific stimulation taking place at the site
11 of infection and presupposes both the expression of viral antigens and of Treg-derived cytokines,
12 such as IL-10 and TGF- β 1 (18, 30, 31). Moreover, circulating tumor associated antigen specific
13 T cells with a regulatory phenotype (CD25^{+(high)}FoxP3⁺) and the ability of secreting IL-10 and
14 exerting suppressive functions have also been described in metastatic melanoma patients (51).

15 Still unclear is the molecular background preferentially favoring the expansion of these cells
16 in patients bearing BKV+ PCa. It is of relevance, however, that prostate tissues appear to be
17 characterized by features consistent with an immunosuppressive microenvironment. Infiltrating
18 CD4⁺ and CD8⁺ T lymphocytes (TIL) are predominantly characterized by regulatory (32, 36) and
19 functionally exhausted (PD-1+, B7-H1+) phenotypes (8, 17, 47). Furthermore, enhanced
20 suppressive function of adaptive CD4⁺ Treg has been observed in the peripheral blood of patients
21 with PCa and found to correlate with metastatic behavior (55).

22 We previously showed that indoleamine-2, 3-deoxygenase (IDO) gene is frequently
23 expressed to high extents in PCa (19). The specific gene product plays a key role in tryptophan

1 metabolism and its enhanced activities might result in both the depletion of an amino acid
2 essential for lymphocyte metabolism and in the generation of toxic metabolites (53). In addition,
3 arginase production by macrophages infiltrating prostatic tissues has been shown to favor the
4 induction of anergy in resident lymphocytes (8). Therefore, in patients bearing a BKV+ PCa,
5 presentation of L-Tag to T cells might occur in conditions likely favoring the generation of
6 immune responses characterized by a high IL-10/IFN- γ gene expression ratio.

7 Most obviously, our data do not allow the postulation of any causal relationship between
8 features of L-Tag specific immune responses, BKV infection and cancer. Nevertheless, they
9 suggest the existence of a complex interaction of potential clinical relevance.

10 Our study has a major limitation. Indeed, although it capitalizes on the analysis of specific
11 immune responses in a substantial number of patients, the number of informative cases, e.g.
12 patients bearing BKV+ lesions with or without BR, is relatively modest. Therefore, additional
13 investigations confirming these results in a larger number of cases are obviously warranted.

14 However, our data provide important novel contributions to the analysis of the increasingly
15 puzzling relationship between BKV infection and PCa by identifying subpopulations of patients
16 deserving major attention in translational research and, most importantly, by highlighting subtle
17 peculiarities of immune responses against BKV L-Tag in patients with PCa, with previously
18 unsuspected associations with the clinical course of this disease.

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33

Figure legends

Figure 1. BKV L-Tag DNA detection in tissue specimens and L-Tag or VP1 specific IgG

response. (A) BKV L-Tag DNA was detectable in either PCa (n=18/43, 42%) or BPH (n=12/38, 32%) surgically excised lesions. Quantitative data, expressed as copy number/ 10^5 cells were comparatively analyzed in positive PCa and BPH tissues. To emphasize the specificity of BKV detection in prostate specimens, JCV L-Tag sequence was also investigated. Rare samples displayed JCV L-Tag sequences either in patients with PCa (n=1/40, 2%) or with BPH (n=1/36, 3%) with copy numbers markedly below 50 copies/ 10^5 cells, the arbitrary limit for BKV L-Tag DNA detection selected in our study (cut-off; dotted line). (B) BKV L-Tag and VP1 IgG titer in patients with either PCa or BPH, stratified according to BKV L-Tag DNA molecular testing in their tissues (positive lesions: BKV+ or +; negative lesions: BKV- or -). Cut-off (dotted line) was set at 0.04, the lowest OD₄₉₂ sufficient to detect antibody activity. Boxes and whiskers (CI 95%) are also reported.

Figure 2. Cytokine gene expression upon *ex vivo* BKV L-Tag peptide-pool stimulation in PBMC from patients bearing PCa, BPH or healthy donors (HD).

(A) IFN- γ (white boxes) and IL-10 (grey boxes) cytokine gene expression was measured upon *ex vivo* BKV L-Tag peptide pool stimulation of PBMC from patients with PCa and PBH, stratified according to their L-Tag specific IgG activity (IgG+ ≥ 0.04). HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta C_t}$)). An arbitrary cut-off was set at 2-fold (dotted line). Boxes and whiskers (CI 95%) were reported to show the significance of differential gene expression for each cytokine based on patients' group stratifications. (B) Correlation (Spearman ρ) between cytokine gene

expression, as detected upon *ex vivo* L-Tag peptide-pool stimulation of PBMC from BKV L-Tag seropositive patients with PCa or BPH and L-Tag specific IgG. Ratios of IL-10/IFN- γ gene expression induced by L-Tag stimulation were plotted against L-Tag specific IgG activity for all IgG+ PCa (n=46; upper quadrant) and IgG+ BPH (n=38; lower quadrant) patients. Cut-off (dotted line) was set at 0.04 OD₄₉₂ (see legend to figure 1). (C) Boxes and whiskers (CI 95%) for IFN- γ (white boxes) and IL-10 (grey boxes) cytokine gene expression, as observed in PBMC from L-Tag IgG+ patients with PCa (n=46), age-matched patients with BPH (n=38) and non age, gender-matched healthy donors (HD; n=8) following *ex vivo* stimulation with BKV L-Tag peptide pool, CMV/EBV/influenza virus peptide pool (CEF) or PHA. HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta C_t}$)). An arbitrary cut-off was set at 2-fold (dotted line).

Figure 3. Cytokine gene expression pattern upon *ex vivo* BKV L-Tag peptide-pool stimulation in L-Tag IgG+ patients bearing BKV+ PCa or BPH. (A) IFN- γ (white boxes) and IL-10 (grey boxes) cytokine gene expression was analyzed upon *ex vivo* BKV L-Tag peptide pool stimulation of PBMC from patients with PCa and BPH, stratified according to their BKV L-Tag DNA molecular testing (positive lesions: BKV+ or +; negative lesions: BKV- or -). HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta C_t}$)). An arbitrary cut-off was set at 2-fold (dotted line). Boxes and whiskers (CI 95%) were reported to show the significance of differences observed for each cytokine gene expression based on patients' group stratifications. (B) IFN- γ (white circles) and IL-10 (white squares) gene expression were measured upon L-Tag peptide-pool stimulation (cut-off 2-fold; dotted line) of PBMC from BKV+/IgG+ patients with PCa (n=17) or BPH (n=6).

(C) IL-10/IFN- γ gene expression ratio observed following L-Tag peptide stimulation of PBMC from BKV+/IgG+ patients bearing BKV+ PCa or BKV+ BPH. Boxes and whiskers (CI 95%) were reported to show differences of cytokine expression pattern between BKV+/IgG+ patients groups.

Figure 4. L-Tag peptide-pool induced cytokine gene expression in L-Tag IgG+ patients bearing BKV+ PCa with or without evidence of biochemical recurrence (BR)

(A) Ratios of IL-10/IFN- γ (grey boxes) and TGF- β 1/IFN- γ (white boxes) gene expression, as detected following L-Tag peptide specific *ex vivo* stimulation of PBMC from L-Tag IgG+ patients bearing BKV+ PCa with (n=7) or without (n=8) BR evidence. HIV peptide-pool stimulation was used as background to compute gene expression fold changes ($2^{-\Delta\Delta C_t}$; cut-off 2-fold). Boxes and whiskers (CI 95%) were reported to show significant differences for both IL-10/IFN- γ and TGF- β 1/IFN- γ gene expression ratios according to BR patients' stratifications. (B) IFN- γ (white bars), IL-10 (light grey bars) and TGF- β 1 (dark grey bars) protein production from 2-week *in vitro* expanded PBMC from L-Tag IgG+ patients bearing BKV+ PCa with (n=7) or without (n=8) BR. HIV peptide-pool *in vitro* stimulation was used as control. Histograms \pm standard errors are reported.

Figure 5. BKV L-Tag-specific expansion of CD4⁺ T cell with a CD25^{+(high)}FoxP3⁺ regulatory phenotype in L-Tag IgG+ patients bearing BR+BKV+ PCa

(A) Representative FACS density plot analysis of *ex vivo* FoxP3 expression in CD4⁺CD25⁺ T cells in PBMC from a PCa patient (upper panel) and a HD (lower panel). The histogram shows means \pm standard errors of *ex vivo* detected frequencies of CD4⁺CD25⁺FoxP3⁺ T cells in L-Tag

IgG+ patients with BR+/BKV+ PCa (dotted bar), BR-/BKV+ PCa (checkered bar) and BKV+ BPH (striped bar), as compared to HD (black bar). **(B)** Gating strategies for CD4⁺ T cell with a CD25^{+(high)}FoxP3⁺ phenotype, as detectable upon peptide stimulation. **(C)** Percentages of CD4⁺CD25^{+(high)}FoxP3⁺ T cells in PBMC from L-Tag IgG+ patients with BR+/BKV+ PCa (n=7), BR-/BKV+ PCa (n=8) or BKV+ BPH (n=7) upon HIV peptide-pool (circles) or L-Tag peptide-pool (squares) stimulation. HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide (triangles). was also used as additional control on BR+/BKV+ PCa (n=6), BR-/BKV+ PCa (n=7) or BKV+ BPH (n=5) L-Tag IgG+ patients. **(D)** Mean Fluorescence Intensity (MFI) of FoxP3 intracellular staining of cultured cells from L-Tag IgG+ patients with BR+/BKV+ PCa, BR-/BKV+ PCa or BKV+ BPH, following HIV peptide-pool (circles), HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide (triangles) or L-Tag peptide-pool (squares) *in vitro* stimulation. Overlaid histograms refer to FoxP3 MFI upon L-Tag peptide-pool and HIV peptide-pool (above) or L-Tag peptide-pool and HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide (below) stimulations, as compared to isotype staining.

Figure 6. Generation of BKV L-Tag specific functional CD4⁺CD25^{+(high)}FoxP3⁺ T cells with effector-memory regulatory phenotype and suppressive activity.

(A) Intracellular IL-10 protein production in CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells from one representative L-Tag IgG+ patient bearing a BKV+ PCa with BR, out of three studied, following L-Tag peptide-pool stimulation (right quadrant), as compared to HCMVpp65₃₄₀₋₃₅₅ promiscuous peptide stimulation (middle quadrant) and to negative control HIV peptide-pool stimulation (left quadrant). **(B)** Expression of the regulatory T cell activation marker CD103 in IL-10-secreting CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells (see panel A) from one representative L-Tag IgG+ patient bearing a BKV+ PCa with BR, out of three tested. Overlaid histograms refer to L-Tag peptide-

1 pool stimulated cells (black line), HCMVpp65₃₄₀₋₃₅₅ stimulated cells (dark gray line) or negative
2 control HIV peptide-pool stimulated cells (light grey line). **(C)** Proliferation index of anti-
3 CD3/CD28 stimulated CD4⁺CD25⁻ T cells from one representative L-Tag IgG⁺ patient bearing a
4 BKV⁺ PCa with BR, out of three tested, co-cultured with autologous L-Tag peptide-pool
5 (circles) or HCMVpp65₃₄₀₋₃₅₅ induced IL-10-secreting CD4⁺CD25^{+(high)}CD127^{-(dim)} T cells
6 (squares), or cultured alone (triangles) over 8 days. WST-1^(OD450) values were plotted against
7 days of incubation in co-cultures performed at a 0.5:1 ratio. **(D)** Mean±SE of proliferation index
8 of anti-CD3/CD28 stimulated CD4⁺CD25⁻ T cells from the three IgG⁺ patients with BR⁺/BKV⁺
9 PCa tested, co-cultured as described above.

10

1 **Table 1: Patients PSA, PCa Gleason score, L-Tag molecular testing and IgG serology**

	PSA level	Gleason score	L-Tag DNA copies/10 ⁵ cells	L-Tag IgG (OD492)		PSA level	L-Tag DNA copies/10 ⁵ cells	L-Tag IgG (OD492)
PCa 1	7.50	7(3+4)	nt	+	BPH 1	12.75	-	+
PCa 2	5.22	9(5+4)	nt	+	BPH 2	na	-	+
PCa 3	4.60	7(4+3)	+	+	BPH 3	4.74	nt	+
PCa 4	5.30	7(4+3)	-	+	BPH 4	na	-	-
PCa 5	4.80	7(3+4)	-	+	BPH 5	na	-	+
PCa 6	10.70	7(3+4)	nt	+	BPH 6	2.96	+	+
PCa 7	7.64	7(4+3)	-	+	BPH 7	na	-	+
PCa 8	5.50	6(3+3)	+	+	BPH 8	18.60	-	+
PCa 9	4.10	7(3+4)	+	+	BPH 9	4.65	nt	+
PCa 10	7.50	8(3+5)	-	+	BPH 10	2.07	-	+
PCa 11	1.60	6(3+3)	nt	-	BPH 11	na	-	+
PCa 12	na	6(3+3)	nt	-	BPH 12	26.00	+	+
PCa 13	7.20	7(3+4)	-	-	BPH 13	1.79	+	-
PCa 14	7.80	7(3+4)	-	+	BPH 14	1.26	-	+
PCa 15	10.80	9(4+5)	nt	+	BPH 15	3.93	-	+
PCa 16	4.78	6(3+3)	+	-	BPH 16	na	-	+
PCa 17	na	na	nt	-	BPH 17	na	nt	+
PCa 18	4.17	9(4+5)	+	+	BPH 18	na	nt	+
PCa 19	25.60	7(4+3)	-	+	BPH 19	na	nt	+
PCa 20	4.70	7(4+3)	-	-	BPH 20	4.40	-	-
PCa 21	5.20	7(3+4)	-	-	BPH 21	17.00	+	+
PCa 22	na	7(3+4)	nt	+	BPH 22	1.16	-	+
PCa 23	4.05	7(3+4)	-	-	BPH 23	6.40	+	-
PCa 24	4.40	7(3+4)	-	-	BPH 24	1.36	+	-
PCa 25	35.00	7(4+3)	+	+	BPH 25	6.34	nt	+
PCa 26	61.30	6(3+3)	-	+	BPH 26	4.8	nt	+
PCa 27	na	na	+	+	BPH 27	10.10	+	+
PCa 28	9.20	7(3+4)	-	-	BPH 28	3.78	-	+
PCa 29	4.80	6(3+3)	-	-	BPH 29	4.26	-	-
PCa 30	na	7(4+3)	nt	+	BPH 30	1.66	+	+
PCa 31	6.59	7(3+4)	-	+	BPH 31	na	+	-
PCa 32	8.73	6(3+3)	nt	+	BPH 32	1.11	-	+
PCa 33	6.60	7(3+4)	+	+	BPH 33	na	-	+
PCa 34	3.44	7(3+4)	+	+	BPH 34	1.67	-	-
PCa 35	6.43	6(3+3)	-	+	BPH 35	na	nt	-
PCa 36	10.00	6(3+3)	nt	+	BPH 36	5.40	-	-
PCa 37	8.80	7(3+4)	+	+	BPH 37	2.12	-	+
PCa 38	7.46	na	nt	+	BPH 38	2.30	-	+
PCa 39	10.90	7(4+3)	+	+	BPH 39	8.14	+	+
PCa 40	106.00	9(4+5)	-	+	BPH 40	na	-	+
PCa 41	na	5(2+3)	-	-	BPH 41	na	nt	+
PCa 42	10.60	7(3+4)	nt	-	BPH 42	4.25	-	-
PCa 43	8.50	6(3+3)	-	+	BPH 43	1.24	-	+
PCa 44	7.20	7(4+3)	-	+	BPH 44	8.90	nt	+
PCa 45	11.52	6(3+3)	+	+	BPH 45	2.66	-	+
PCa 46	2.44	9(4+5)	+	+	BPH 46	2.30	+	-
PCa 47	na	na	nt	+	BPH 47	2.85	nt	+
PCa 48	48.00	7(4+3)	+	+	BPH 48	3.50	+	+
PCa 49	na	6(3+3)	+	+	BPH 49	0.36	nt	+
PCa 50	na	7(3+4)	-	+	BPH 50	15.70	-	+
PCa 51	na	na	nt	+				
PCa 52	4.70	7(3+4)	-	+				
PCa 53	2.56	6(3+3)	+	+				
PCa 54	6.78	7(3+4)	nt	+				
PCa 55	4.40	7(3+4)	-	+				
PCa 56	6.50	6(3+3)	-	-				
PCa 57	4.50	7(4+3)	+	+				
PCa 58	4.60	6(3+3)	+	+				
PCa 59	206.00	9(4+5)	-	+				
PCa 60	4.11	6(3+3)	nt	+				
	n=50	n=55	n=43	n=60		n=36	n=38	n=50
			BKV+ n=18	IgG≥0.04 n=46			BKV+ n=12	IgG≥0.04 n=38
			BKV - n=25	IgG<0.04 n=14			BKV - n=26	IgG<0.04 n=12

na: not available

nt: not tested

L-Tag BKV+ : evidence of L-Tag DNA detection in tissue specimens

L-Tag IgG+ : OD₄₉₂ ≥0.04

in bold: patients sharing L-Tag BKV+ specimens and IgG+ activity against L-Tag (IgG≥0.04): PCa n=17/18, BPH n=7/12; p=0.005 (χ²)

1 **Table 2: Analysis of L-Tag IgG+ patients bearing BKV+ PCa with or without BR**

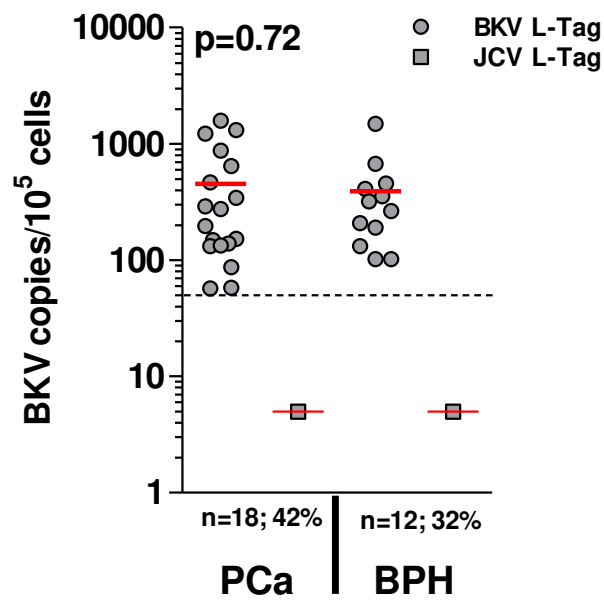
<i>Patients</i>	<i>BR*</i> (PSA ng/ml)	<i>L-Tag DNA</i> (copies/10 ⁵ cells)	<i>L-Tag IgG</i> (OD 452)	<i>Patients</i>	<i>BR*</i> (PSA ng/ml)	<i>L-Tag DNA</i> (copies/10 ⁵ cells)	<i>L-Tag IgG</i> (OD 452)
<i>IgG+/BKV+/BR+** PCa n=7</i>				<i>IgG+/BKV+/BR+*** PCa n=8</i>			
PCa 2	6	nt	+	PCa 9	-	+	+
PCa 8	6	+	+	PCa 16	-	+	-
PCa 18	6	+	+	PCa 25	-	+	+
PCa 27	96	+	+	PCa 33	-	+	+
PCa 37	6	+	+	PCa 34	-	+	+
PCa 48	48	+	+	PCa 39	-	+	+
PCa 49	24	+	+	PCa 46	-	+	+
PCa 57	6	+	+	PCa 53	-	+	+
PCa 60	6	nt	+	PCa 58	-	+	+
<i>IgG+/BKV+ BPH n=7</i>							
BPH 6	na	+	+				
BPH 12	na	+	+				
BPH 21	na	+	+				
BPH 27	na	+	+				
BPH 30	na	+	+				
BPH 39	na	+	+				
BPH 48	na	+	+				

nt: not tested
na: not available
**: biochemical recurrence (BR) upon 5-year follow up.*
*** : BR+ (PSA ≥ 0.2 ng/ml)*
**** : BR- (PSA < 0.04 ng/ml)*
in bold: timing of biochemical recurrence expressed in weeks

2

Figure 1

A



B

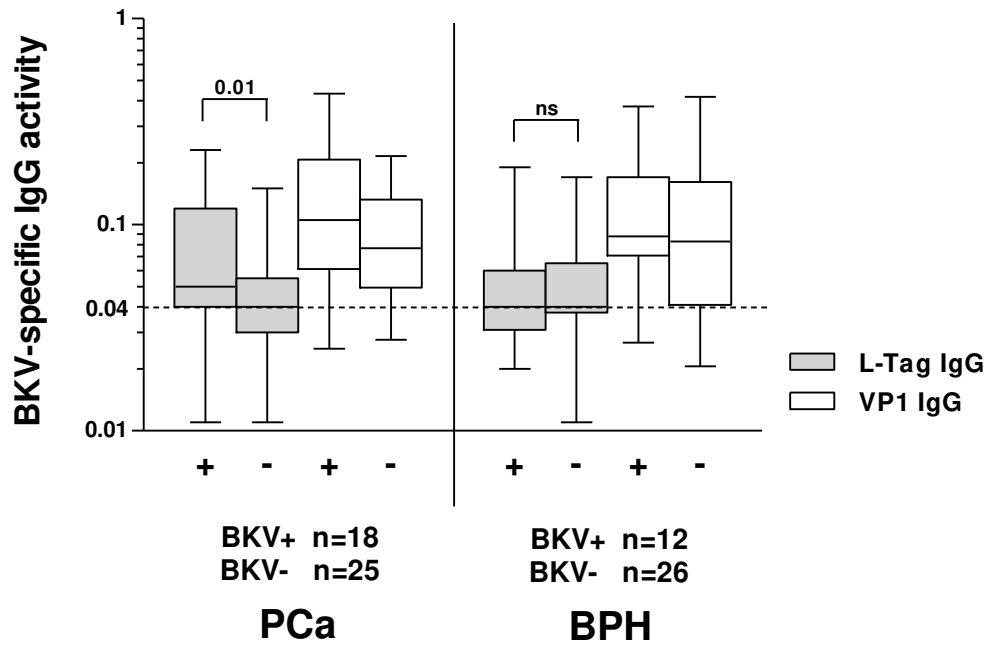


Figure 3

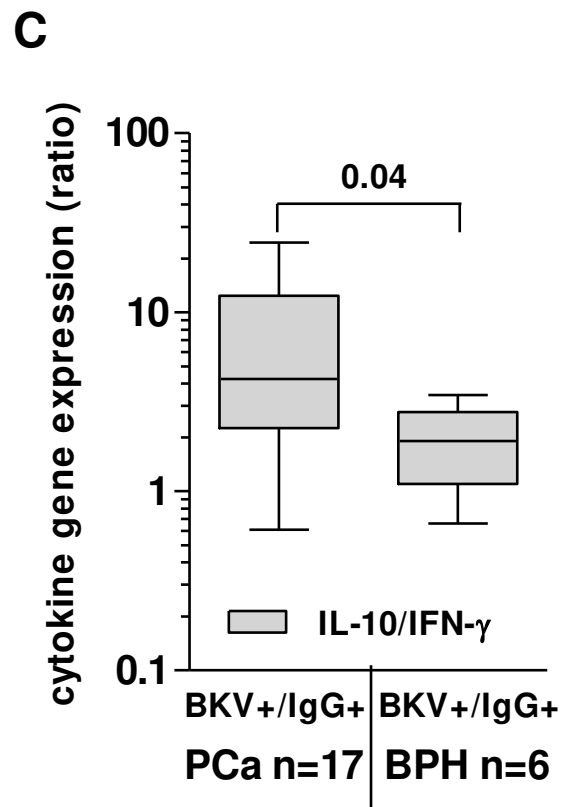
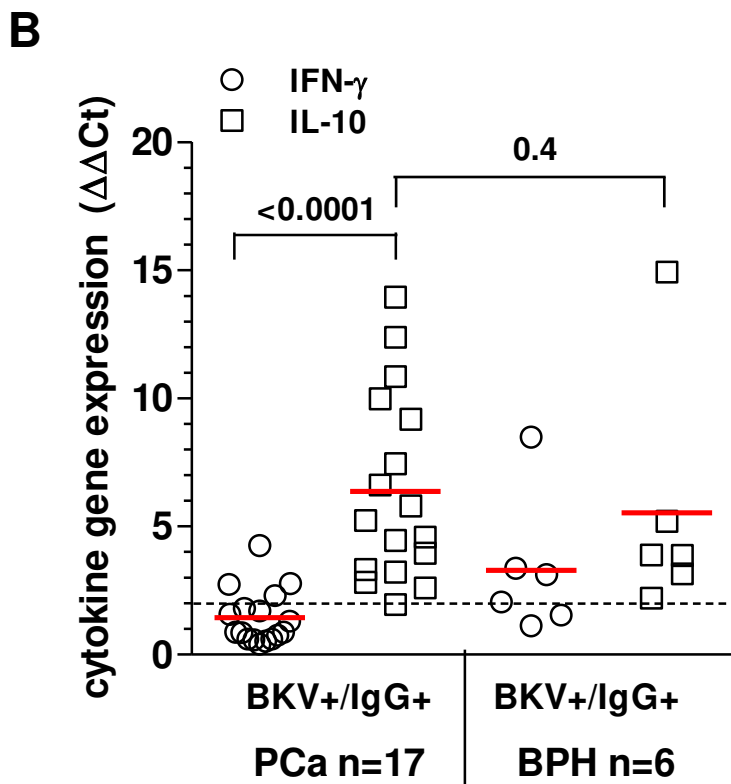
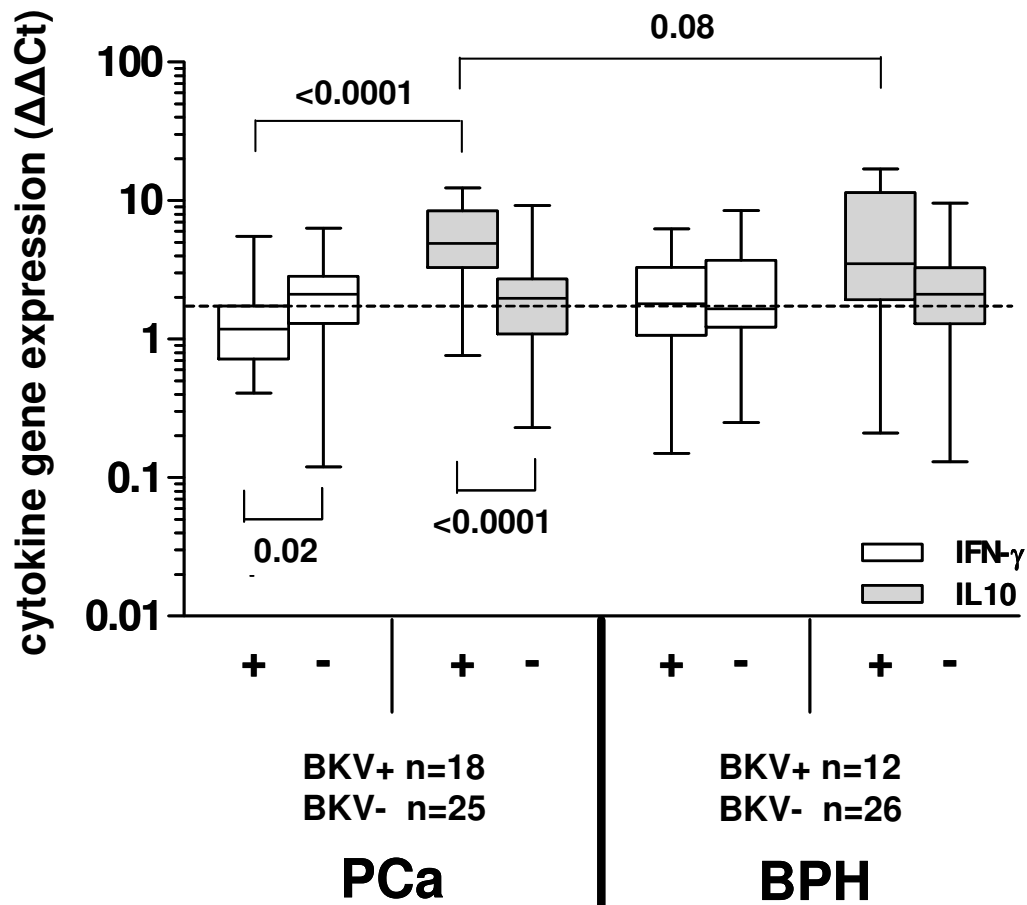
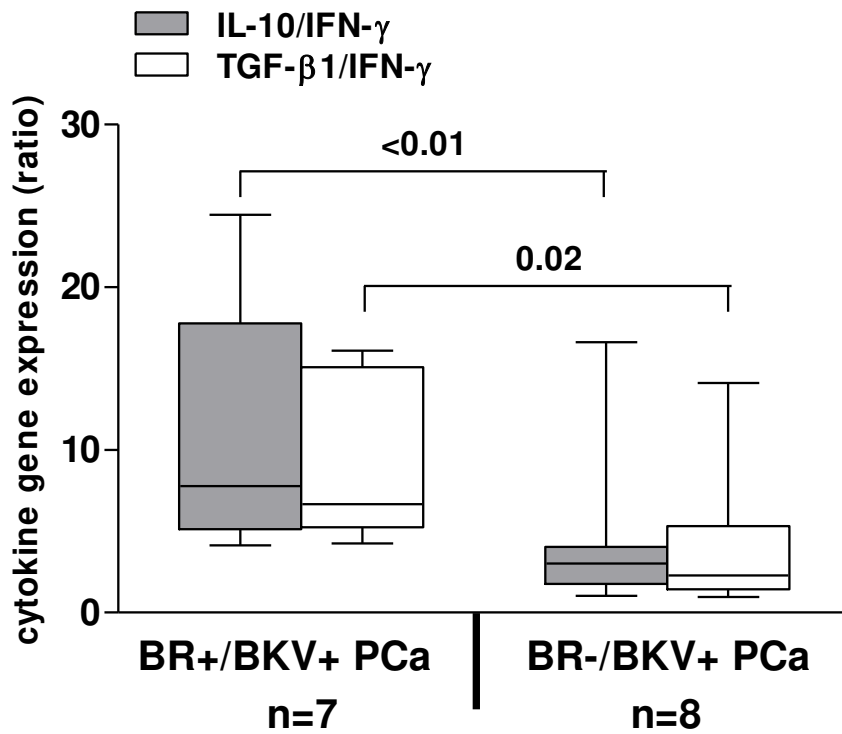
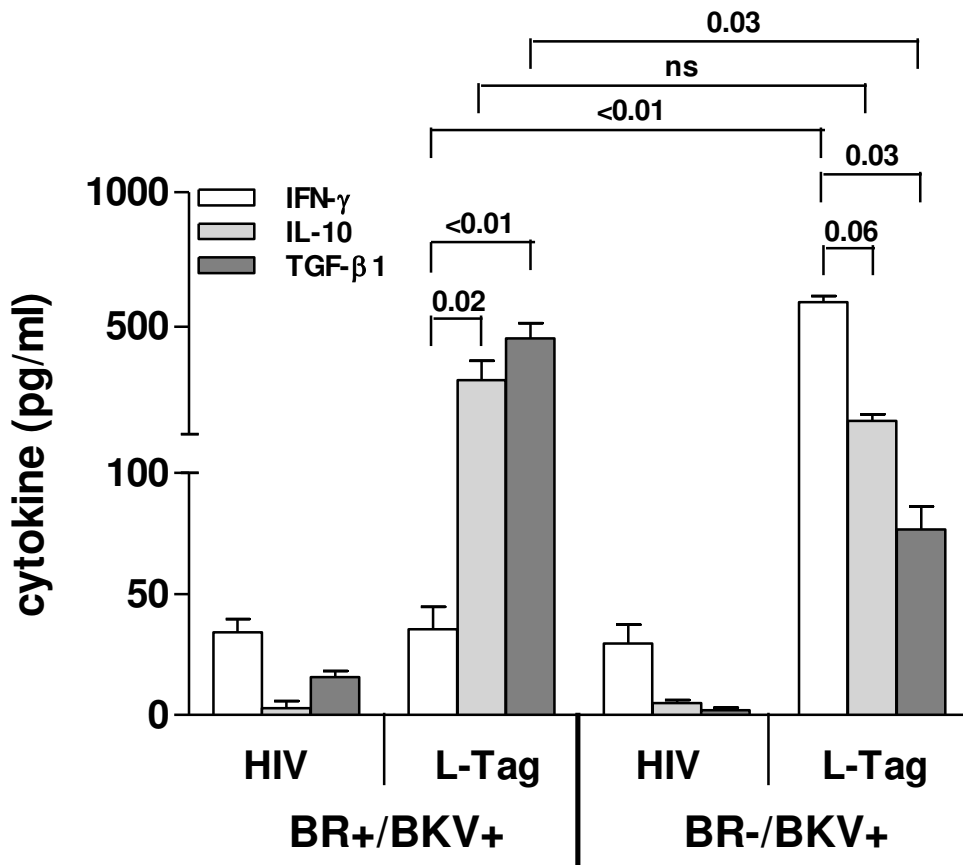


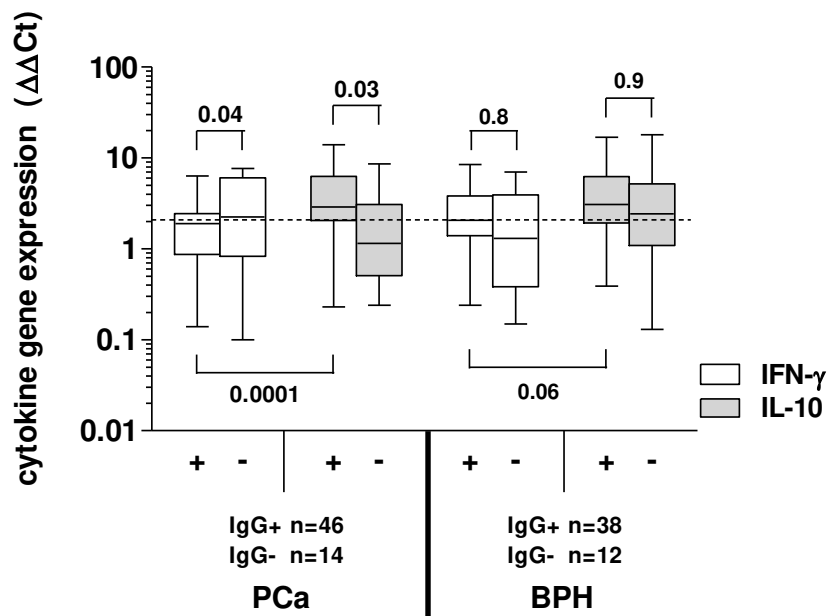
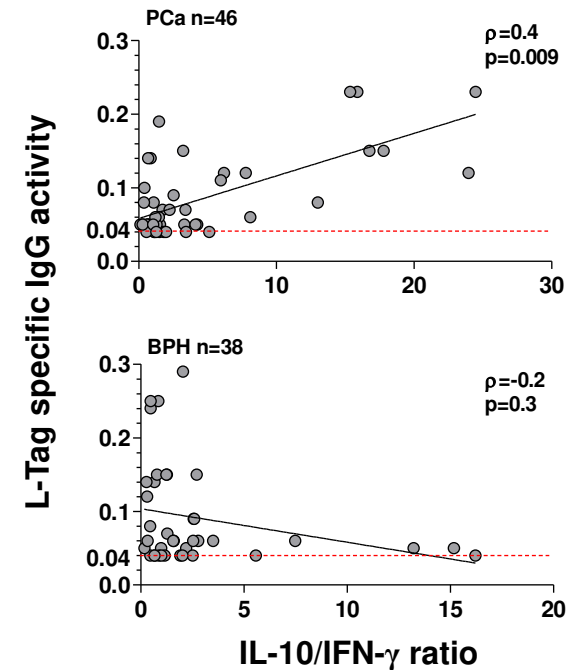
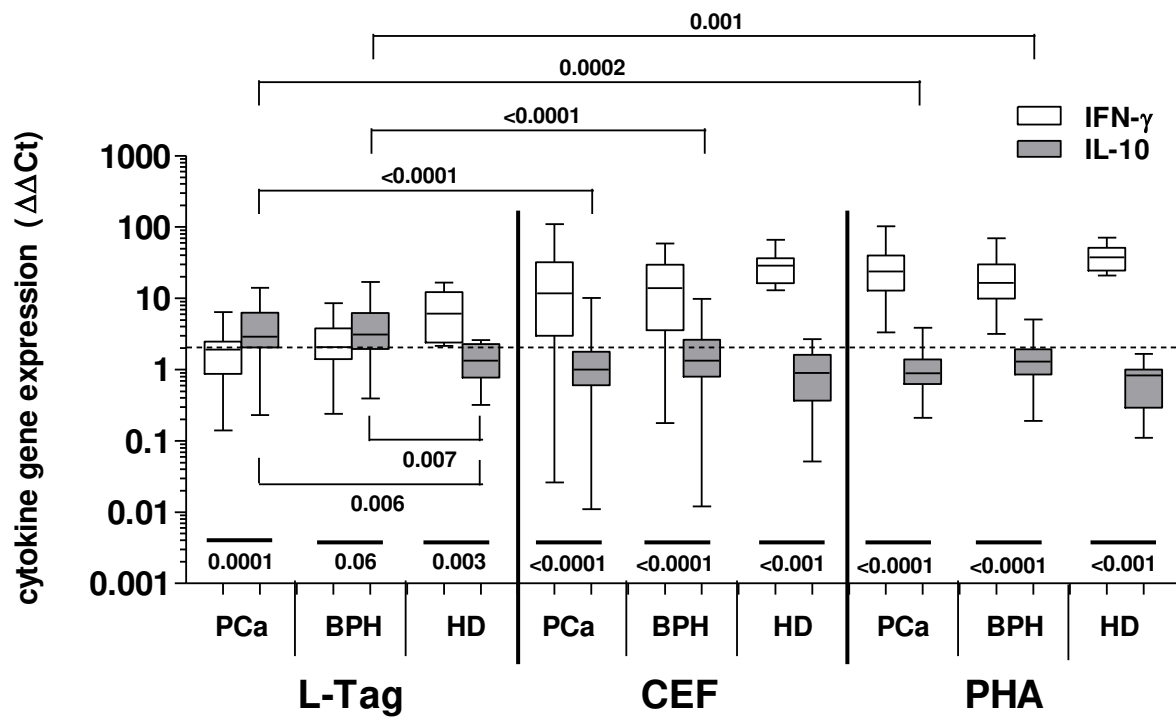
Figure 4

A



B



A**B****C****Figure 2**

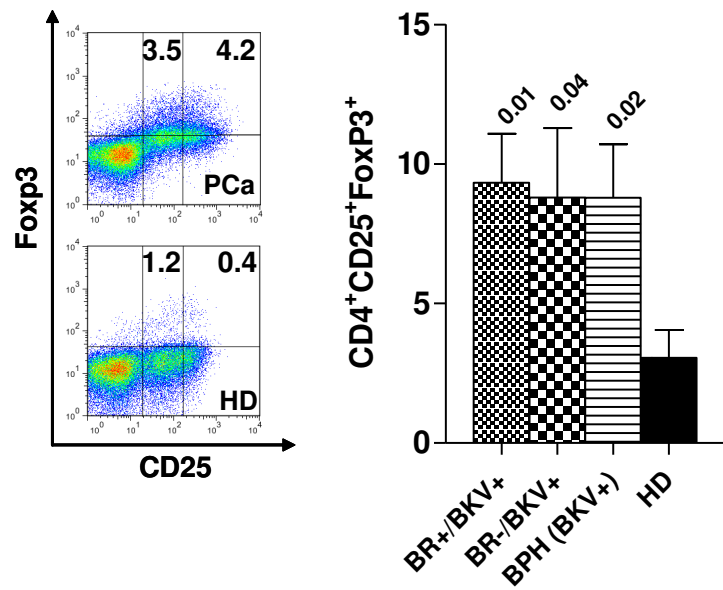
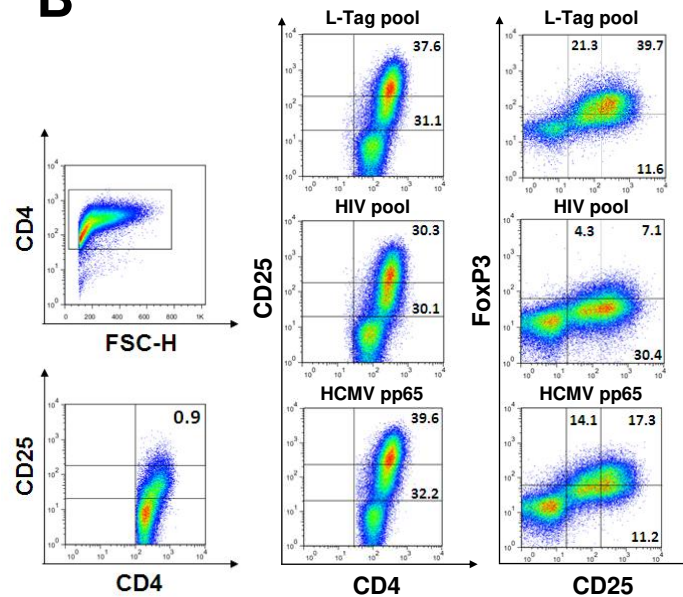
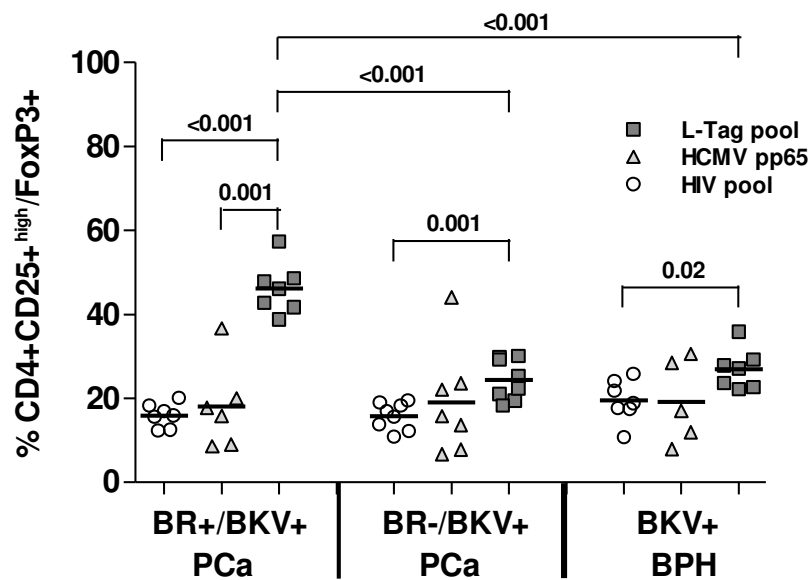
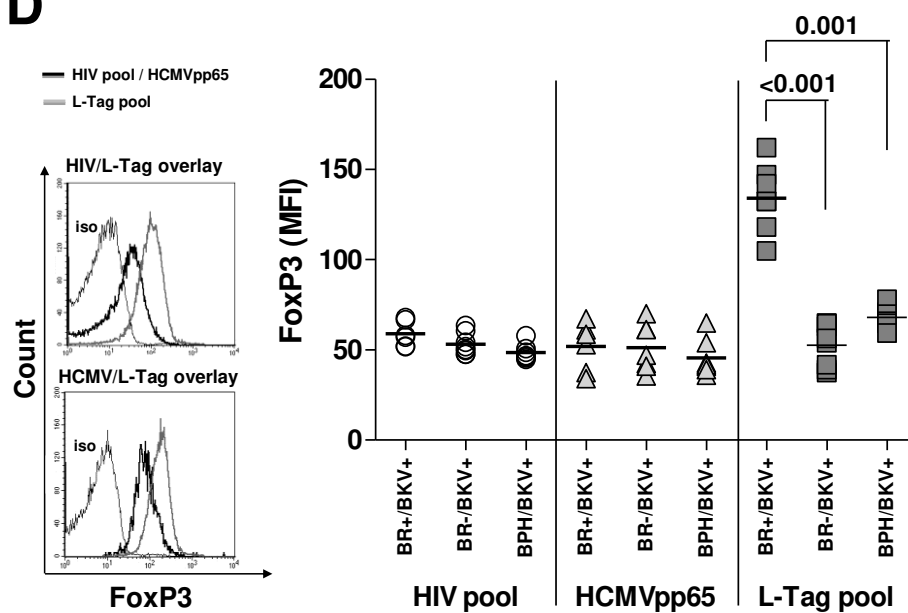
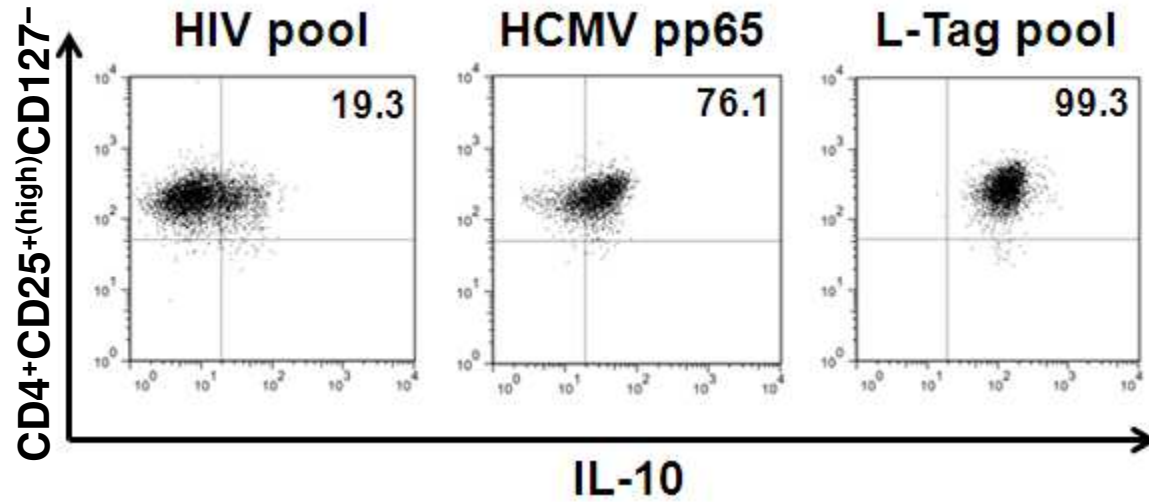
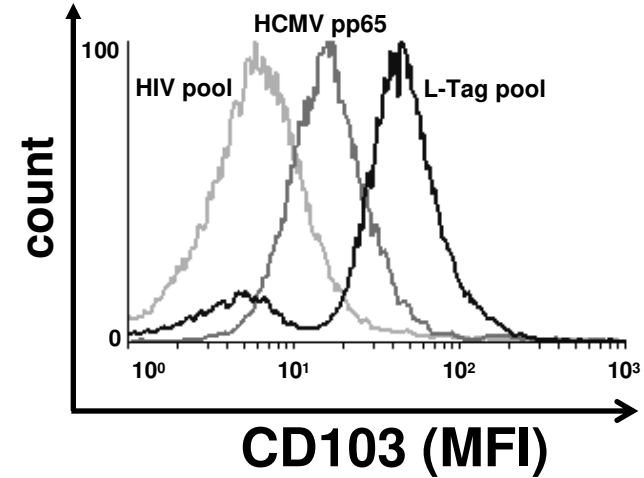
A**B****C****D****Figure 5**

Figure 6

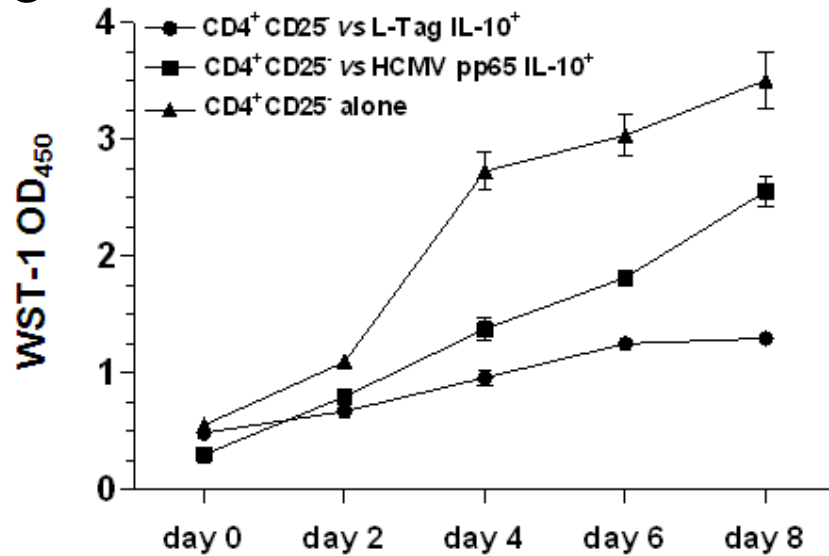
A



B



C



D

